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Limiting Fluoroquinolone Resistance in *S. aureus* in an In Vitro Model Using Optimized Treatment Intensity and Duration

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AMENDED ABSTRACT

Background:
An in vitro pharmacodynamic infection model assessed garenoxacin (GRN) therapy intensity and duration in preventing *S. aureus* resistance (R) emergence. Phenotypic and genotypic parameters of FQ-R were evaluated on sequential isolates recovered during a clinically relevant 10-day interval.

Methods:
Doses and schedules of GRN were evaluated with multiple R assessments made on *S. aureus* ATCC 25923 over 10 days. At periodic intervals (20), the strain was recovered and tested against 3 FQs using CLSI microdilution methods. Replicate testing was performed with and without 2 efflux pump inhibitors (EPI; 20 µg/ml reserpine [RES] or phe-arg-ß-napththylamide [PABN]). All strains were analyzed by PCR for mutations in the quinolone-resistance determining region (QRDR; *gyrA, gyrB, grlA* and *grlB*).

Results:
EPI effects on *S. aureus* are in the Table.

	Norfloxacin (NOR) MIC µg/ml			Ciprofloxacin (CIP) MIC µg/ml			Garenoxacin (GRN) MIC µg/ml		
Strain	Alone	+ RES	+ PABN	Alone	+ RES	+ PABN	Alone	+ RES	+ PABN
ATCC 25923	2	0.25	0.5	0.5	0.12	0.12-0.25	0.03	0.03	0.015-0.03
SA 6	1	0.5	0.5	0.5	0.12	0.25	0.06	0.06	0.06
SA 11	0.5	0.25	0.25	0.25	0.06	0.12	0.06	0.06	0.06
SA 19	1	0.5	0.5	0.5	0.12	0.25	0.06	0.06-0.12	0.12

The initial GRN MIC for *S. aureus* was 0.03 µg/ml which increased only 2- to 4-fold (0.06-0.12; mode, 0.12 µg/ml) over 10 days. No mutations were detected in the QRDR. RES showed significant efflux pump inhibition with NOR (2- to 8-fold MIC decreases) and CIP (4-fold), as did PABN (2- to 4-fold for both). EPI had minimal effect on lowering GRN MIC values (0- to 2-fold).

Conclusions:
GRN-R in isolates from R plates was not due to target site alteration, but rather to efflux pump expression. An optimized regimen suppressed resistance completely. The pathway to FQ-R is likely initiated by efflux pump over-expression with some, but not all agents (GRN). Identification of optimal regimens for existing FQs is critical, potentially extending their useful clinical life by suppressing emergence of R subpopulations.

INTRODUCTION

The intensity of antimicrobial therapy has been shown in both *in vitro* and animal model systems to have a profound influence on the amplification of antimicrobial resistant subpopulations of microorganisms that pre-exist in a larger bacterial population. Little attention has been paid to the effect of therapy duration on resistant subpopulations.

Previously, we conducted a hollow fiber infection model (HFIM) study of garenoxacin against a strain of methicillin-susceptible *Staphylococcus aureus* (ATCC 25923). As part of that evaluation, we ascertained drug exposures that would suppress the amplification of resistant mutants over the duration of the experiment (21 days). We calculated that an AUC/MIC ratio of 100 would suppress resistant mutants for a period of 2 days (the usual duration of HFIM studies) and would fail around day 4-5. We also calculated that it would take considerably greater drug exposure (AUC/MIC ratio of 280) to suppress resistant mutants for a period of 10 days, the therapeutic duration most commonly seen for serious infections.

We subsequently performed a prospective validation study, examining regimens of AUC/MIC ratios of 100 and 280. As predicted, the larger exposure suppressed amplification for the full 10 days and the regimen of an AUC/MIC ratio of 100 failed at day 5 with breakthrough growth. Resistant mutants were recovered from plates containing three times the baseline MIC over the full 10 days. It was the aim of this study to examine these mutants and to ascertain the mechanism of resistance that had allowed survival of these isolates.

MATERIALS AND METHODS

Experimental protocol. Doses and schedules of garenoxacin were evaluated with multiple resistance assessments made on *S. aureus* ATCC 25923 over 10 days. At periodic intervals (20 total), the strain was recovered from the HFIM and tested against three quinolone agents using broth microdilution methods. Replicate testing was performed with and without 2 efflux pump inhibitors. All strains were analysed by PCR for mutations in the quinolone-resistance determining region (QRDR).

Susceptibility Test Methods. Strains were tested by the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method (M7-A7 [2006]) using validated commercially prepared panels (TREK Diagnostics, Cleveland, OH) in cation-adjusted Mueller-Hinton broth against two quinolone agents: ciprofloxacin (Sigma-Aldrich, Inc., St. Louis, MO) and garenoxacin (kindly supplied by Schering-Plough, Kenilworth, NJ). Norfloxacin, ciprofloxacin and garenoxacin were subsequently tested in freshly prepared broth panels with and without known efflux pump inhibitors (reserpine and phe-arg-ß-napthylamide or PABN [Sigma-Aldrich]) that were incorporated into the test medium at a final concentration of 20 µg/ml. *Staphylococcus aureus* ATCC 29213 was included as a quality control (QC) strain when performing susceptibility testing of the quinolone agents; QC results for the three agents were within CLSI specified ranges.

PCR amplification of the QRDR. All strains were analyzed for mutations in the QRDR region. Amplification of topoisomerase IV and gyrase A gene segments responsible for fluroquinolone resistant phenotypes was carried out as described by Horii et al (2003). The primer sets utilized included: grlA-F, GAT GAG GAG GAA ATC TAG; grlA-R, GTT GGA AAA TCG GAC CTT; grlB-F, GAC AAT TGT CTA AAT CAC TTG TG; grlB-R, CAT CAG TCA TAA TAA TTA CAC; gyrA-F, GCG ATG AGT GTT ATC GTT GCT; gyrA-R, CAG GAC CTT CAA TAT CCT CC; gyrB-F, CAG CGT TAG ATG TAG CAA GC; gyrB-R, CGA TTT TGT GAT ATC TTG CTT TCG. PCR products were cleaned using the QIAquick PCR purification kit (QIAGEN GmbH, Germany).

Sequencing of QRDR amplicons. Generated amplicons were sequenced using the Sanger-based dideoxy sequencing strategy involving the incorporation of fluorescent dye-labeled terminators into the sequencing reaction products. Sequences obtained were subjected to NCBI BLAST search to detect the presence of any mutations in the QRDR region. QRDR sequences of *S. aureus* ATCC 25923 were used as a control.

RESULTS

- Using an optimized garenoxacin treatment regimen to prevent resistance emergence in the HFIM, MIC values for the three quinolone agents when testing *S. aureus* ATCC 25923 varied from baseline only two- to four-fold over the sampling period of 10 days; no strain expressed overt resistance (Table 1).
- No mutations were detected in the QRDR including *gyrA, gyrB, grlA* and *grlB*.
- Reserpine showed significant efflux pump inhibition with norfloxacin (two- to eight-fold MIC decreases) and ciprofloxacin (four-fold), as did PABN (two- to four-fold for both).
- Efflux pump inhibitiors had minimal effect on lowering garenoxacin MIC values (zero- to two-fold).
- Breakthrough growth of less susceptible mutants at three-fold the baseline MIC when using a HFIM regimen of AUC/MIC ratio of 100 appears to be caused by increased expression of efflux pumps alone.

Table 1. In vitro activity of three fluoroquinolones without and with efflux pump inhibitors (reserpine [RES] and phe-arg-ß-naphthylamide [PABN]) against *S. aureus* ATCC 25923 recovered daily following continuous exposure to garenoxacin in the hollow-fiber infection model.

	MIC values in µg/ml								
	Norfloxacin			Ciprofloxacin			Garenoxacin		
ATCC 25923	Alone	+ RES	+ PABN	Alone	+ RES	+ PABN	Alone	+ RES	+ PABN
Day 1; Baseline	2	0.25	0.5	0.5	0.12	0.12-0.25	0.03	0.03	0.015-0.03
Day 2				0.5			≤0.03		
Day 3				0.5			0.06		
Day 4				0.5			0.06		
Day 5				0.5			≤0.03		
Day 6	1	0.5	0.5	0.5	0.12	0.25	0.06	0.06	0.06
Day 7				0.5			0.06		
Day 8				0.5			≤0.03		
Day 9				1			0.12		
Day 10				0.25			≤0.03		
Day 11	0.5	0.25	0.25	0.25	0.06	0.12	0.06	0.06	0.06
Day 12				0.5			≤0.03		
Day 13				0.5			≤0.03		
Day 14				0.5			≤0.03		
Day 15				0.5			0.06		
Day 16				0.5			≤0.03		
Day 17				0.5			0.06		
Day 18				0.5			0.06		
Day 19	1	0.5	0.5	0.5	0.12	0.25	0.06	0.06-0.12	0.12
Day 20				0.25			≤0.03		
Day 21				0.5			0.06		

CONCLUSIONS

- Garenoxacin resistance in isolates from resistance plates was not due to target site alteration, but rather to efflux pump expression. An optimized regimen suppressed the emergence of QRDR mutational resistance.
- The pathway to fluoroquinolone resistance is likely initiated by efflux pump over-expression with some (norfloxacin, ciprofloxacin), but not all agents (garenoxacin).
- Identification of optimal regimens for existing fluoroquinolones is critical, potentially extending their useful clinical therapeutic role by suppressing emergence of stable resistant subpopulations.

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